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**Decreased met-enkephalin-induced current in locus coeruleus neurons from G protein-gated inwardly rectifying potassium channel knockout mice.** . Society for Neuroscience Abstracts, (2001) Vol. 27, No. 2, pp. 2146. print. Meeting Info.: 31st Annual Meeting of the Society for Neuroscience. San Diego, California, USA. November 10-15, 2001. ISSN: 0190-5295. ; English

**G PROTEIN - GATED POTASSIUM ( GIRK ) CHANNELS CONTRIBUTE TO THE REINFORCING EFFECTS OF COCAINE.** . Society for Neuroscience Abstract Viewer and Itinerary Planner, (2002) Vol. 2002, pp. Abstract No. 500.7. <http://sfn.scholarone.com>. cd-rom. Meeting Info.: 32nd Annual Meeting of the Society for Neuroscience. Orlando, Florida, USA. November 02-07, 2002. Society for Neuroscience. ; English

**Hyperalgesia and blunted morphine analgesia in G protein-gated potassium channel subunit knockout mice.** . Neuroreport, (2002 Dec 20) 13 (18) 2509-13. Journal code: 9100935. ISSN: 0959-4965.; English

**G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons.** . Journal of neuroscience : official journal of the Society for Neuroscience, (2002 Jun 1) 22 (11) 4328-34. Journal code: 8102140. ISSN: 1529-2401.; English

Michael C. Wilson  
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# Hyperalgesia and blunted morphine analgesia in G protein-gated potassium channel subunit knockout mice

Cheryl L. Marker, Stephanie C. Cintora, Maria I. Roman, Markus Stoffel<sup>1</sup> and Kevin Wickman<sup>CA</sup>

Department of Pharmacology, University of Minnesota, 6-120 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455; <sup>1</sup>Laboratory of Metabolic Diseases, The Rockefeller University, New York, NY 10021, USA

<sup>CA</sup>Corresponding Author: wickm002@tc.umn.edu

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Our aim was to determine whether G protein-gated potassium (Kir3) channels contribute to thermonociception and morphine analgesia. Western blotting was used to probe for the presence of Kir3.1, Kir3.2, Kir3.3, and Kir3.4 subunits in the mouse brain and spinal cord. Hot-plate paw-lick latencies for wild-type, Kir3.2 knockout, Kir3.3 knockout, and Kir3.4 knockout mice were measured at 52°C and 55°C, following the s.c. injection of either saline or 10 mg/kg morphine. Paw-lick latencies for Kir3.4 knockout mice were similar to those of wild-type mice, consistent with the restricted expression pattern of Kir3.4 subunit in the mouse brain. In contrast, Kir3.2 knockout and Kir3.3 knockout mice displayed hyperalgesia at both temperatures tested, and both Kir3.2 knockout and Kir3.3 knockout mice displayed shorter paw-lick latencies

following morphine administration, with Kir3.2 knockout mice exhibiting the more dramatic phenotype. Kir3.2/Kir3.3 double knockout mice displayed a greater degree of hyperalgesia than either the Kir3.2 knockout or Kir3.3 knockout mice, while performing similarly to Kir3.2 knockout mice following morphine administration. We conclude that G protein-gated potassium channels containing Kir3.2 and/or Kir3.3 play a significant role in responses to moderate thermal stimuli. Furthermore, the activation of Kir3 channels containing the Kir3.2 subunit contributes to the analgesia evoked by a moderate dose of morphine. As such, receptor-independent Kir3 channel agonists may represent a novel and selective class of analgesic agent. *NeuroReport* 13: 2509–2513 © 2002 Lippincott Williams & Wilkins.

**Key words:** GIRK; Hot-plate; Kir3; Nociception; Opioid

## INTRODUCTION

Morphine administration elicits a wide range of effects in mammals, including analgesia, respiratory depression, nausea, tolerance, and physical dependence [1]. Mouse knockout studies have identified the key role for the mu opioid receptor in many of these effects of morphine, although the activation of delta and kappa opioid receptors probably also contributes to the effects [2]. Activation of mu, delta, or kappa opioid receptors triggers, via pertussis toxin-sensitive G proteins, the inhibition of adenylyl cyclase and voltage-gated calcium channels, and activation of G protein-gated inwardly-rectifying potassium (Kir3/GIRK) channels, among other effects [1]. The relative contributions of these and possibly other enzymes and ion channels to the behavioral effects of opioid receptor activation are poorly understood.

G protein-gated potassium channels are homo- and heterotetrameric complexes formed by Kir3 channel subunits [3]. Of the four known mammalian Kir3 subunit genes (Kir3.1–4), three (Kir3.1–3) exhibit abundant and widespread expression patterns in the brain [4,5]. Studies in hetero-

logous expression systems have demonstrated that the functional properties of Kir3 channels are largely independent of subunit composition, with the notable exception that Kir3.1 homomultimers do not form functional channels due to failure to achieve cell surface expression [6,7].

Four lines of evidence suggest that Kir3 channels are *bona fide* effectors in opioid signaling pathways in the nervous system. First, the expression of Kir3.1, Kir3.2, and Kir3.3 has been detected in many of the key CNS targets of morphine, including the locus coeruleus and periacqueductal gray [4,5]. Second, the mu, delta, and kappa opioid receptors can activate recombinant Kir3 channels in heterologous expression systems [8,9]. Third, the activation of Kir3-like conductances by opioid receptor agonists has been reported in a variety of neurons [10,11]. Indeed, we demonstrated recently that the opioid-induced current in mouse locus coeruleus neurons is mediated primarily by the activation of Kir3 channels containing Kir3.2 and Kir3.3 [12]. Finally, *weaver* mutant mice, which harbor a mutant Kir3.2 gene, exhibited blunted morphine-induced analgesia in tests of thermonociception [13].

Here, we utilized knockout mice lacking one or more Kir3 subunits to directly test the hypothesis that intact G protein-gated potassium channel function is required for the analgesic effect of morphine. Thermonociception and the analgesic effect of morphine were measured in Kir3 subunit knockout and wild-type mice using the hot-plate test. Our findings indicate that Kir3 channels contribute significantly to both thermonociception and the analgesic effect of morphine.

## MATERIALS AND METHODS

**Western blotting:** Isolation and Western blotting of brain and spinal cord membrane proteins from wild-type and Kir3 subunit knockout mice were performed as described [12]. Protein concentrations were determined using a Lowry protein assay after trichloroacetic acid precipitation (Sigma Diagnostics; St. Louis, MO). Anti-Kir3.1, Kir3.2, and Kir3.3 (Alomone Laboratories; Jerusalem, Israel) were used according to manufacturer's specifications. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus; Amersham Pharmacia Biotech; Piscataway, NJ) and a Storm 840 phosphorimager.

**Subjects:** The use of animals for this study was approved by the University of Minnesota Institutional Animal Care and Use Committee (Protocol 0011A74121). Laboratory and housing facilities were accredited by the American Association for the Accreditation of Laboratory Animal Care (Assurance of Compliance #A3456-01). Efforts were made to minimize the pain and discomfort of the animals throughout the course of this study. The generation of Kir3.2 knockout, Kir3.3 knockout, Kir3.4 knockout, and Kir3.2/Kir3.3 double knockout mice has been described previously [12,14,15]. Null Kir3 genes were back-crossed through 7–14 rounds against the C57BL/6J mouse strain prior to beginning this study. Efforts were made to balance the wild-type and knockout groups for each temperature and drug condition with respect to gender and age (8–10 weeks). Group sizes ranged from 13–49 and 5–17 animals for the 52°C and 55°C studies, respectively. Mouse genotypes were determined by PCR analysis of tail DNA samples as described [15].

**Hot-plate test:** The hot-plate test was performed on three consecutive days between 13.30 and 16.30 h. On days 1 and 2, mice were transferred to a testing room, injected s.c. with an 0.9% saline solution (10 µl/g), placed in the ambient temperature hot-plate apparatus (Columbus Instruments; Columbus, OH) for 7 min, and then returned to their home cage and housing room. On day 3, mice were given an injection of either saline (10 µl/g) or 10 mg/kg morphine sulfate (Sigma Chemicals; St. Louis, MO), and returned to their housing cages for 30 min to allow for maximal development of the morphine analgesic effect [16]. The mice were then placed on the hot-plate apparatus warmed to either 52 ± 0.1°C or 55 ± 0.1°C, and the latency to first paw-lick was recorded to the nearest second by an observer blinded to subject genotype. To eliminate the potentially confounding effects of prior drug exposure and learning in the hot-plate test, distinct cohorts of drug-naïve animals were used for each drug (saline and morphine) and

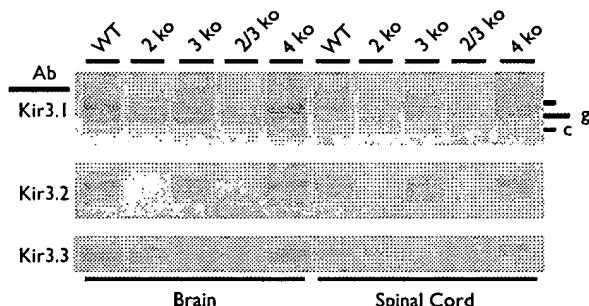
temperature condition. Mean paw-lick latencies were analyzed by linear regression, followed by pairwise comparisons using the Student's *t*-test (SPSS software package; SPSS Inc.; Chicago, IL). Significance was set at *p* < 0.05.

## RESULTS

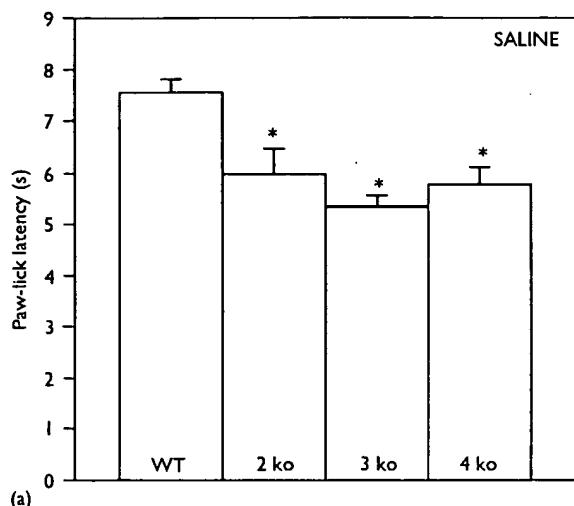
Western analysis of membrane protein extracts from adult wild-type and Kir3 knockout mouse brain and spinal cord tissue is shown in Fig. 1. Kir3.1, Kir3.2, and Kir3.3 were clearly detected in wild-type mouse brain samples. Lower levels of each subunit were also observed in spinal cord, with the level of Kir3.3 approaching the lower limit of detection. We were unable to detect expression of Kir3.4 in either tissue (data not shown). Previous studies have demonstrated that Kir3.1 is not heavily-glycosylated and fails to achieve surface membrane localization in the absence of Kir3.2, Kir3.3, or Kir3.4 [12,15,17]. Given the nearly complete loss of heavily glycosylated Kir3.1 in spinal cord tissue from Kir3.2 knockout mice, and the relatively low level of Kir3.3 expression in wild-type spinal cord, it is likely that Kir3.2 is the most prominent functional Kir3 subunit in the mouse spinal cord.

Thermonociception and morphine-induced analgesia were assessed by the hot-plate test using wild-type, Kir3.2 knockout, Kir3.3 knockout, Kir3.4 knockout, and Kir3.2/Kir3.3 double knockout (52°C only) mice as behavioral subjects. The time to first paw-lick was measured at 55°C or 52°C, following the s.c. injection of either saline or a moderate dose (10 mg/kg) of morphine. Mean paw-lick latencies were analyzed by linear regression using paw-lick latency as the dependent variable and hot-plate temperature, drug status, genotype, and gender as independent variables. Drug status (*p* < 0.001), hot-plate temperature (*p* < 0.001), and mouse genotype (*p* = 0.001), but not gender (*p* = 0.530), were significant predictors of performance in this test. Since gender was not found to be a significant predictor of paw-lick latency, data from male and female mice were combined to increase the power of the study.

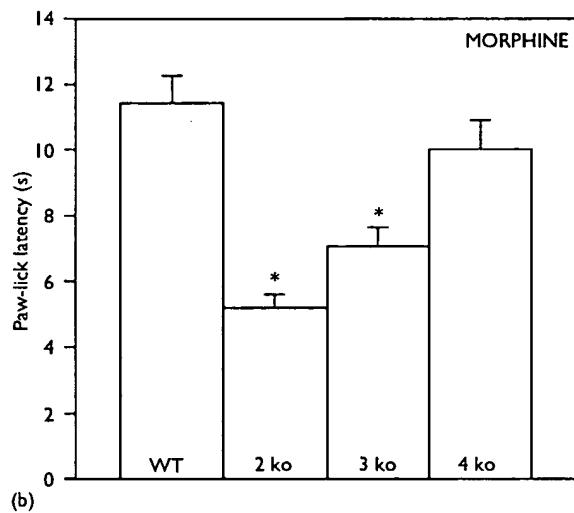
Mean paw-lick latencies (± s.e.m.) at 55°C for saline-treated Kir3.2 knockout (6.0 ± 0.5 s; *p* < 0.05), Kir3.3 knockout (5.4 ± 0.2 s; *p* < 0.05), and Kir3.4 knockout (5.8 ± 0.3 s;



**Fig. 1.** Western blot analysis of Kir3 subunit expression in adult mouse brain and spinal cord. Whole brain and spinal cord tissues were harvested from wild-type (WT) mice and mice lacking Kir3.2 (2 ko), Kir3.3 (3 ko), Kir3.4 (4 ko), or both Kir3.2 and Kir3.3 (2/3 ko) subunits. Ten µg of membrane protein was loaded per lane. The immunoreactive pattern for Kir3.1 is consistent with previous studies that characterized heavily-glycosylated (h), glycosylated (g), and core (c) versions of Kir3.1 [17].



(a)

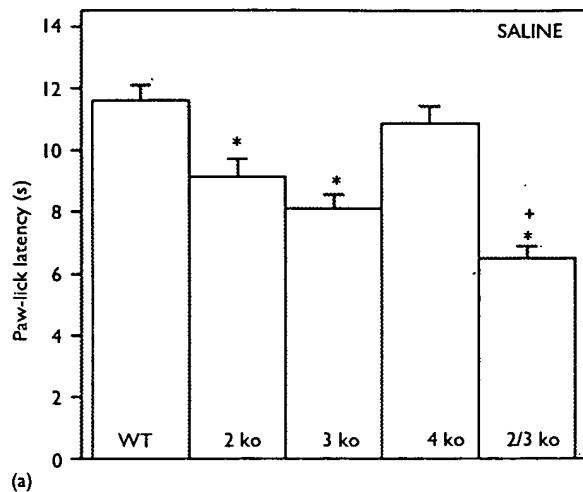


(b)

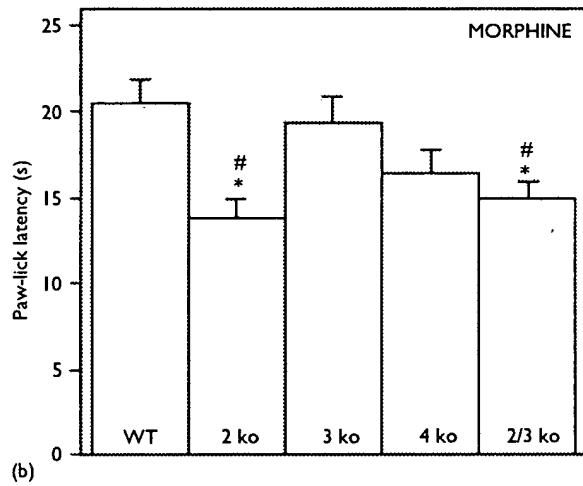
**Fig. 2.** Mean paw-lick latencies ( $\pm$  s.e.m.) of wild-type (WT) mice and mice lacking Kir3.2 (2 ko), Kir3.3 (3 ko), or Kir3.4 (4 ko) subunits at 55°C, following injection of either saline (a) or 10 mg/kg morphine (b). \* $p$  < 0.005 vs WT.

$p$  < 0.05) mice were all significantly lower than that observed for wild-type mice ( $7.6 \pm 0.3$  s; Fig. 2a). Mean paw-lick latencies in separate cohorts of morphine-treated (10 mg/kg) mice are shown in Fig. 2b. We observed no difference in performance between morphine-treated Kir3.4 knockout ( $10.1 \pm 0.9$  s) and wild-type mice ( $11.5 \pm 0.85$  s;  $p = 0.253$ ). In contrast, mean paw-lick latencies of morphine-treated Kir3.2 knockout ( $5.2 \pm 0.4$  s;  $p < 0.001$ ) and Kir3.3 knockout ( $7.1 \pm 0.5$  s;  $p < 0.001$ ) mice were significantly shorter than observed for wild-type mice. Although there was an observed increase in paw-lick latency after morphine treatment (compared to saline treatment) within the wild-type ( $p < 0.001$ ), Kir3.3 knockout ( $p = 0.003$ ), and Kir3.4 knockout ( $p < 0.001$ ) test groups, the paw-lick latencies of saline- and morphine-treated Kir3.2 knockout mice were indistinguishable ( $p = 0.252$ ).

Mean paw-lick latencies at 52°C for saline-treated wild-type ( $11.6 \pm 0.5$  s) and Kir3.4 knockout ( $10.9 \pm 0.6$  s) mice



(a)



**Fig. 3.** Mean paw-lick latencies ( $\pm$  s.e.m.) of wild-type (WT) mice and mice lacking Kir3.2 (2 ko), Kir3.3 (3 ko), Kir3.4 (4 ko), and both Kir3.2 and Kir3.3 (2/3 ko) subunits at 52°C, following injection of either saline (a) or 10 mg/kg morphine (b). \* $p$  < 0.005 vs WT;  $^+$  $p$  < 0.05 vs saline-treated Kir3.2 knockout and Kir3.3 knockout groups;  $\#$  $p$  < 0.05 vs morphine-treated Kir3.2 knockout mice.

were equivalent ( $p = 0.418$ ; Fig. 3a). In contrast, saline-treated Kir3.2 knockout ( $9.2 \pm 0.6$  s;  $p = 0.004$ ) and Kir3.3 knockout ( $8.1 \pm 0.5$  s;  $p < 0.001$ ) mice exhibited significantly shorter paw-lick latencies than observed for the wild-type group. Interestingly, Kir3.2/Kir3.3 double knockout mice displayed marked hyperalgesia at 52°C ( $6.5 \pm 0.4$  s;  $p < 0.05$ ), an effect significantly greater than that observed for either Kir3.2 or Kir3.3 single subunit knockout group. We observed no significant difference in paw-lick latencies between morphine-treated wild-type ( $20.5 \pm 1.4$  s), Kir3.3 knockout ( $19.3 \pm 1.6$  s;  $p = 0.566$ ), and Kir3.4 knockout ( $16.4 \pm 1.4$  s;  $p = 0.118$ ) mice at 52°C (Fig. 3b). In contrast, morphine-treated Kir3.2 knockout ( $13.7 \pm 1.2$  s) and Kir3.2/Kir3.3 double knockout ( $14.9 \pm 1.1$  s) mice exhibited equivalent ( $p = 0.453$ ) latencies, both of which are significantly shorter than observed for wild-type mice ( $p < 0.001$ ).

## DISCUSSION

In this study, we examined the contribution of G protein-gated potassium channels to thermonociception and morphine-induced analgesia. We measured the effect of Kir3 subunit ablation on thermonociception and analgesia induced by a moderate dose (10 mg/kg) of morphine using the hot-plate test. We observed that mean paw-lick latencies for mice lacking one or two Kir3 subunits at two different temperatures (52° and 55°C) and for two different drug conditions (morphine and saline) were invariably lower than the latencies of wild-type control groups. We demonstrated that Kir3.2, Kir3.3, and Kir3.4 knockout mice exhibited a range of hyperalgesic phenotypes at both temperatures. In addition, mice lacking the *Kir3.2* gene (Kir3.2 knockout and Kir3.2/Kir3.3 double knockout mice) displayed severely blunted responses to morphine. These observations argue strongly that G protein-gated potassium channels contribute to both thermonociception and morphine-induced analgesia.

The effects on thermonociception and morphine-induced analgesia were mildest in the Kir3.4 knockout mice, consistent with previous studies demonstrating a restricted expression pattern for Kir3.4 mRNA in the rodent brain [5,18]. Nevertheless, Kir3.4 knockout mice displayed slightly lower paw-lick latencies than wild-type animals at both temperatures and for both drug conditions. At 55°C, the difference between saline-treated wild-type and Kir3.4 knockout mice reached the level of statistical significance. While these findings were somewhat unexpected, it is possible that Kir3.4-containing channels in discrete neuronal populations in the brain and/or spinal cord could contribute to thermonociception. Although we were unable to detect Kir3.4 protein in either brain or spinal cord samples by Western blotting, Kir3.4 mRNA was detected in a handful of neuron populations in the rodent brain, including the endopiriform nucleus and claustrum of the insular cortex, the globus pallidus, parafascicular and paraventricular thalamic nuclei, and the ventromedial hypothalamic nucleus [18]. It remains to be determined whether Kir3.4 is found at appreciable levels in the spinal cord.

The main findings from this study complement and extend those of a previous study involving *weaver* mice, which harbor a point mutation in the Kir3.2 pore domain [19,20]. *weaver* mice exhibited decreased morphine-induced analgesia in the hot-plate and tail-flick tests [21]. Interpretation of these findings, however, is complicated by the significant developmental abnormalities associated with the *weaver* mutation, including a virtual absence of cerebellar granule cells and dramatic degeneration of straital dopaminergic neurons [22]. Furthermore, G protein-gated potassium channels containing the *weaver* Kir3.2 protein exhibit altered ion selectivity and decreased sensitivity to G protein  $\beta\gamma$  subunits [19,20]. Given that Kir3.2 knockout mice do not exhibit the developmental abnormalities associated with the *weaver* mutation and harbor a clean loss-of-function of the *Kir3.2* gene, we felt that this mutant mouse line offered a better opportunity to assess the effects of Kir3.2-containing channels on thermonociception and morphine-induced analgesia.

Comparisons between the performances of the Kir3.2 knockout, Kir3.3 knockout, and Kir3.2/Kir3.3 double knockout mice offer some insight into the possible mechanisms underlying the observed phenotypes. Both the Kir3.2

knockout and Kir3.3 knockout mice exhibited comparable degrees of hyperalgesia at both temperatures tested, and the simultaneous loss of both Kir3.2 and Kir3.3 appeared to represent a behavioral summation of the phenotypes of the single subunit knockout animals. In contrast, the mean paw-lick latency of morphine-treated Kir3.2/Kir3.3 double knockout was equivalent to that of Kir3.2 knockout mice while Kir3.3 knockout mice were not different from wild-type mice. These observations are similar to the findings from our recent electrophysiological analysis of locus coeruleus neurons taken from mice lacking Kir3.2 and/or Kir3.3 [12], where we observed that the loss of Kir3.2 correlated more strongly with decreased opioid-induced current than the loss of Kir3.3. Although the precise mechanism(s) remains to be determined, both studies argue that the Kir3.2 subunit plays an important role in opioid signaling.

Moderate thermal stimuli increase the firing of afferent neurons, which in turn increase the firing of ascending neurons in the dorsal horn of the spinal cord [23]. Descending monoaminergic neurons originating in the periaqueductal gray and rostroventral medulla, project to the dorsal horn of the spinal cord, where they can inhibit signal transmission between primary afferent and ascending neurons. Morphine administered systemically relieves the tonic inhibition of descending neurons by hyperpolarizing interneurons in the periaqueductal gray and rostroventral medulla [23]. At present, however, we do not know whether the hyperalgesia or blunted analgesic effect of morphine observed in the knockout mice results from a loss of Kir3 function in supraspinal or spinal pain processing regions, or in both. Indeed, we demonstrated that the Kir3 channel subunits Kir3.1, Kir3.2, and Kir3.3 are found in both mouse brain and spinal cord. It is also not possible to discern whether the blunted effect of morphine reflects a direct disruption of opioid signaling or a disruption of other signaling systems triggered by opioids. In this regard, it should be noted that  $\alpha_2$ -adrenergic and serotonin 5-HT1A receptors have been linked to Kir3 channel activation *in vivo* [10]. Future studies involving intrathecal and intracerebroventricular drug administration coupled with the use of selective receptor agonists should help to clarify these issues.

## CONCLUSION

We present evidence that G protein-gated potassium channel activation contributes significantly to thermonociception and the analgesic effect of morphine. As such, the Kir3 channel class could constitute a novel target for pharmacologic strategies designed to manage pain. Interestingly, recent studies have shown that ethanol and membrane-permeable local anesthetics can activate Kir3 channels in a receptor-independent fashion [24,25]. Thus, it may be possible to design compounds targeting Kir3 channels with analgesic effects comparable to morphine and without its associated, undesirable side effects.

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